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Award Number: W81XWH-04-1-0462

TITLE: BRCA1 Regulation of Fanconi Anemia Proteins in DNA Damage Repair

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REPORT DATE: May 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-05-2006	Annual	2 Apr 2005 - 1 Apr 2006

4. TITLE AND SUBTITLE
BRCA1 Regulation of Fanconi Anemia Proteins in DNA Damage Repair

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0462

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Brigham and Women's Hospital
Boston, MA 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: BRCA1 is strongly associated with the breast cancer. BRCA1 associates with numerous proteins that repair DNA damage. Fanconi Anemia (FA) is a rare autosomal recessive disorder. It has been shown that BRCA1 regulates one of FA proteins, called FANCD2, by a process called ubiquitination. However, exactly how the FA proteins and BRCA1 interact to regulate DNA damage repair obscure. In this project, we hypothesize that BRCA1 ubiquitination of FANCD2 is affected by association with the FANCA protein complex and by association with DNA damage when embedded in chromatin. Specific aims are that (1) does BRCA1 monoubiquitinate FANCD2 in vivo using purified ubiquitination factors? (2) Do embedding FA proteins in chromatin affect their function as ubiquitination substrates? (3) Is the ubiquitination of FA proteins by BRCA1 affected by binding to damaged DNA? During the first year of grant, we purified the FANCD2, FANCA and BRCA1/BARD1 from baculovirus-infected insect cells and we identified that BRCA1 could ubiquitinated FAND2, dependent on the E2 enzymes. Also, we found direct DNA binding activity of the FANCD2 protein. Now we are trying to know the function of FANCD2 and modified FANCD2 (ubiquitinated FANCD2) on DNA or chromatin. Finally, we are trying to understand the relationship between BRCA1 and FANCD2 in the DNA damage repair pathway by keeping this project.

15. SUBJECT TERMS
Breast Cancer, BRCA1, FA, FANCD2, Ubiquitination, Deubiquitination

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	8	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

In this project, we study the inter-relationship of BRCA1 and FANCD2, and we have made considerable progress toward completion of the specific aims. BRCA1 is the breast-specific tumor suppressor protein 1. FANCD2 is the Fanconi Anemia protein D2. Both proteins execute vital functions in the repair of DNA damage, but how this occurs is unknown. In this project, we hypothesize that BRCA1-dependent ubiquitination activity modifies FANCD2, and the resulting change in the FANCD2 causes a change in its activity and affects the DNA repair process.

Specific aims in our project are
(1) Does BRCA1 monoubiquitinate FANCD2 in vivo using purified ubiquitination factors?
(2) Do embedding FA proteins in chromatin affect their function as ubiquitination substrates?
(3) Is the ubiquitination of FA proteins by BRCA1 affected by binding to damaged DNA?

Body

Task 1: Purification of FANCD2, BRCA1, and associated factors, and testing whether BRCA1 ubiquitinates FANCD2.

Status: Completed

We purified the FANCA, FANCD2, BRCA1, and BARD1 proteins from recombinant baculovirus infected insect cells exactly as originally proposed (Figure. 1).

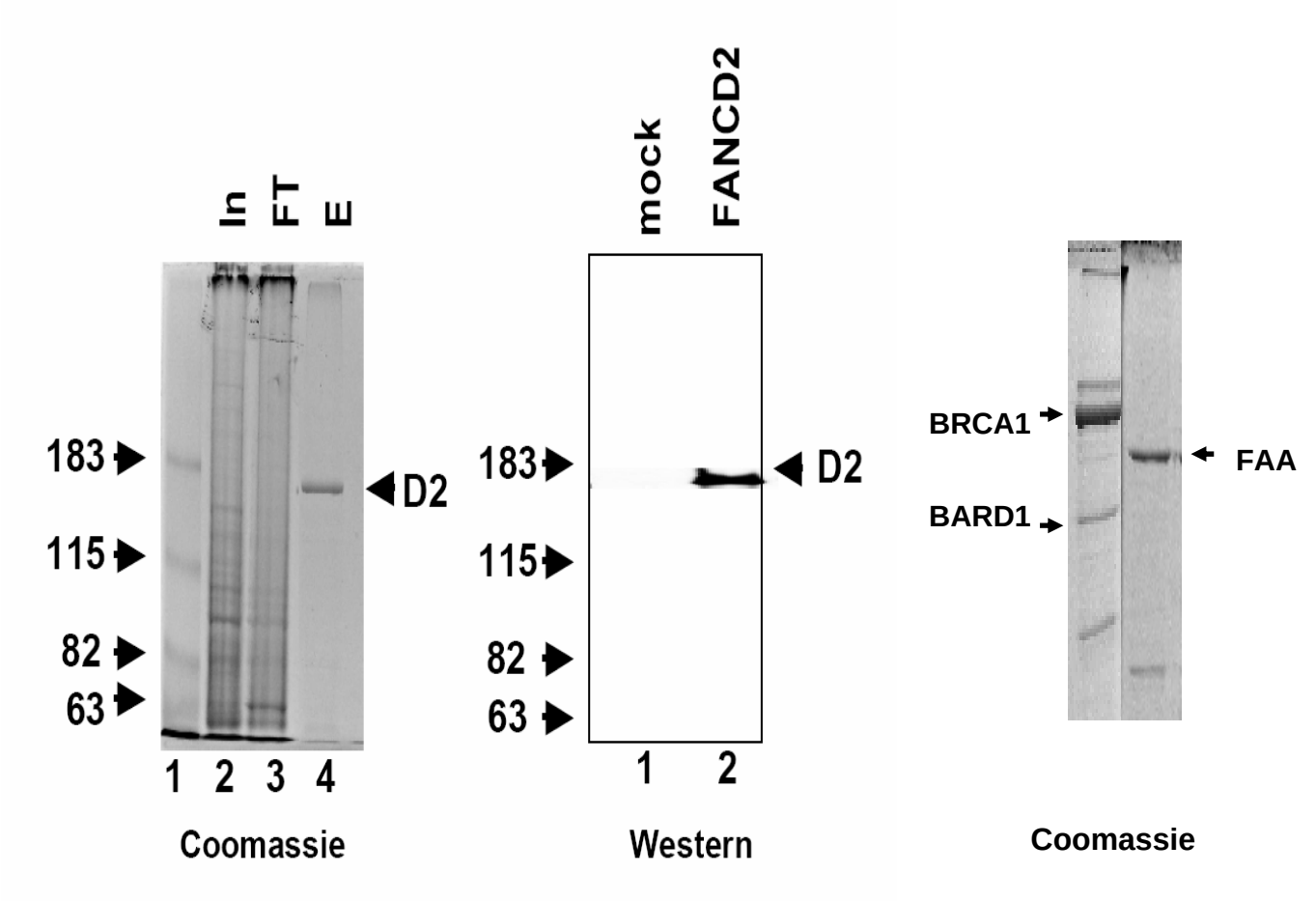

Figure 1. FANCD2, FANCA and BRCA1

Purified proteins were subjected to SDS-PAGE analysis. FANCD2 protein migrated at 160 kDa (left panel), consistent with its predicted mass, and immunoblot analysis revealed that this polypeptide was indeed FANCD2 (middle panel). FANCA and BRCA1/BARD1 were also purified from baculovirus-infected insect cells and analyzed by SDS-PAGE and Coomassie stain.

Next, we undertook the in vitro ubiquitination assay to ascertain ubiquitination system dependent on the BRCA1/BARD1. Consistent with our hypothesis, we found that BRCA1 ubiquitinated FANCD2 dependent on the specific E2 ubiquitin conjugating enzyme (Ubc; Figure. 2). BRCA1/BARD1 (B/B)

proteins ubiquitinated the FANCD2 protein dependent on UBCH5a or Ubc6. However, to our surprise, BRCA1 was not required for the ubiquitination of FANCD2 in the presence of UbcH5c. In this last case, the ubiquitination was independent of the E3 ligase. FANCA had minimal effect on the ubiquitination reaction (not shown). We conclude that the BRCA1 has some important roles in the ubiquitination of FANCD2 in response to the DNA damage, but it is not essential.

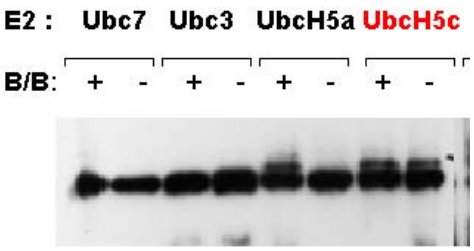

Figure 2. FANCD2 ubiquitination by B/B

Task 2: Is ubiquitination of FANCD2 by BRCA1 affected by embedding FANCD2 in chromatin?
Status: in progress.

Due to results in Task 3, this specific aim will be completed after aim 3.

Task 3: Is the ubiquitination of FA proteins by BRCA1 affected by binding to damaged DNA?

Status: In progress.

We have made considerable progress in this aim. We hypothesized that the FANCD2 protein binds to DNA in a structure-specific fashion. The structures would exist at sites of DNA damage. Since Holliday junction DNA is a potential intermediate in the homologous recombination pathway, we tested this structure first. In an electrophoretic mobility shift assay (EMSA), full-length FANCD2 retarded the mobility of a 125-bp Holliday junction DNA probe (four 30 bp arms; Figure 3). Three different recombinant protein preparations from baculovirus-infected cells were analyzed. FANCD2 was compared to FANCA and to a mock purification from empty baculovirus-infected cells. FANCD2 (40 nM) bound to the Holliday junction DNA and migrated in a diffuse shift that formed a band below the origin of the gel. As a positive control, the BRCA1/BARD1 could bind to the Holliday junction DNA probe well in our system. FANCA protein did not bind to the Holliday junction DNA probe at the same concentration of FANCD2 protein. The shifted bands in the reaction with FANCA were also present in the mock purification from the cells infected with empty virus indicating that these complexes were low level, nonspecific contaminants.

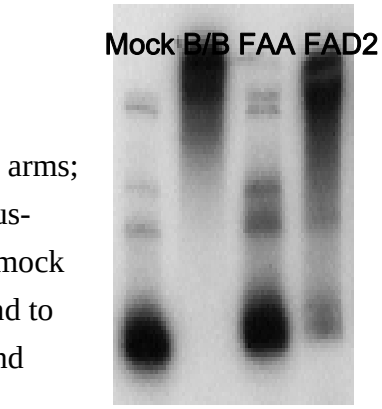

Figure 3. FANCD2 binds DNA.

FANCD2 was included in DNA binding reactions at different concentrations (Figure. 4). We observed diffuse bands that migrated progressively slower in reactions with higher FANCD2 concentrations. This

finding was most consistent with the interpretation that the protein-DNA complex contained multiple FANCD2 molecules. Since the stoichiometry of DNA binding could not be determined, we did not determine a K_d for the complex. Instead, we estimated binding affinity by the concentration of FANCD2 protein that bound half of the probe. Approximately half of the Holliday junction DNA probe was bound in reactions containing 15 nM FANCD2.

The specificity of DNA binding was determined using various probes and DNA competitors. FANCD2 bound to 65-bp double-stranded linear DNA (Figure. 4) and to a 65 bp Y shaped DNA (data not shown). When using a 65 bp linear DNA probe, 50% of the probe was bound at about 20 nM FANCD2, indicating that the binding affinity of FANCD2 to linear 65 bp was modestly decreased relative to the 125 bp Holliday junction probe. By competition analysis (Figure 4B), we found that

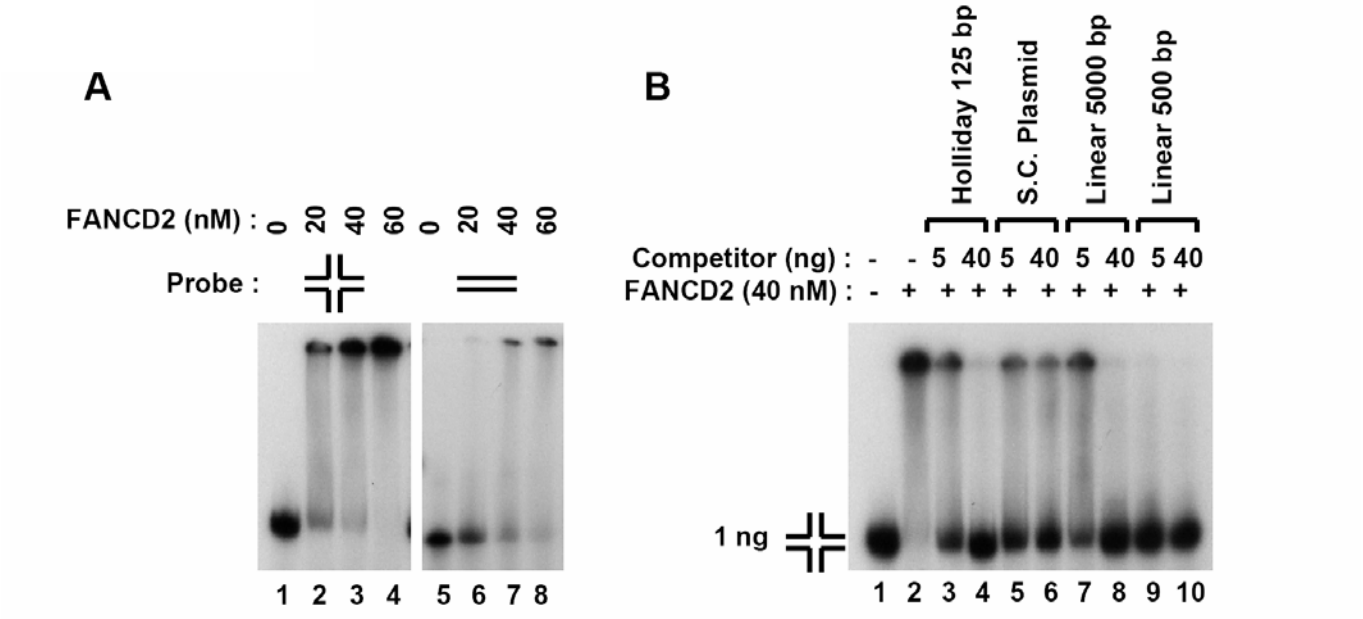

Figure 4. FANCD2 binds to various DAN forms

FANCD2 bound to Holliday junctions and to DNA ends. Surprisingly, ends on short DNA molecules did not bind to FANCD2 (not shown), but DNAs containing an end and about 500 bp DNA were effective in binding.

Plans for this task will include the analysis of how ubiquitination by BRCA1 affects the DNA binding activity of FANCD2.

Key Research Accomplishments

1. Purification of FANCA and FANCD2 from insect cells.
2. Making the FANCA and FANCD2 stable cell lines.
3. Establishment of in vitro ubiquitination using the BRCA1/BARD, E1, E2, ubiquitin, FANCD2
4. Understanding that BRCA1 is an important for FANCD2 function but is not essential for ubiquitination of FANCD2
5. Finding the direct DNA binding activity of FANCD2 protein

Reportable Outcomes

1. The manuscript entitled “Direct DNA binding activity of the Fanconi Anemia D2 protein” has been accepted for publication in J Biol Chem.

Conclusions

During the first year of the award from the Department of Defense Breast Cancer Research Program, we have established the in vitro ubiquitination assay using the BRCA1/BARD1, E1, E2 and FANCD2 of substrate. We found that BRCA1 ubiquitinated the FANCD2 protein dependent on the E2 enzymes (UBCH5a and UBCH6) in vitro. However, from published data, another enzyme is required for the ubiquitination of FAND2 in a cell. Surprisingly, the ubiquitination of FANCD2 in vitro could occur independent of any E3 ligase. FANCA did not stimulate or repress the ubiquitination of FANCD2. Taken together, although BRCA1 has some important roles in the ubiquitination of FANCD2 in response to the DNA damage, it is not essential. Since FANCD2 functions in the nucleus and regulates the repair of DNA damage, we tested whether the FANCD2 protein binds to DNA. This study reveals that the FANCD2 protein binds to Holliday junction DNA and to DNA ends. This is the first biochemical activity identified for this key protein in the Fanconi Anemia pathway. This activity is consistent with a role for the FANCD2 protein in the repair of double stranded DNA breaks. However, it is unclear how phosphorylation and ubiquitination of FANCD2 regulate this activity and more work will be required to determine how the FANCD2 protein functions in the repair of DNA damage. Therefore, in future work we will test whether the FANCD2 protein functions in the repair of DNA damage. In the mean while, we also try to know the BRCA1 function in regulation of FANCD2 or cooperation with FANCD2 in the DNA damage response, especially breast cancer cell lines.